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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANTS:	Jonathan M. Rothberg, et al.		
ASSIGNEE:	CuraGen Corporation		
SERIAL NUMBER:	09/814,338	EXAMINER:	Young J. Kim
FILING DATE:	March 21, 2001	ART UNIT:	1637
FOR:	METHOD OF SEQUENCING A NUCLEIC ACID		

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF MARCEL MARGULIES, Ph.D. UNDER 37 C.F.R. §1.132

I, MARCEL MARGULIES, declare and state that:

1. I am Vice President of Engineering, at 454® Life Sciences, the exclusive licensee of this application. My previous employment includes Director of New Technology Research at Perkin-Elmer's Instrument Division in Norwalk, CT, and Associate Director of the Hubble Space Telescope project.

2. I earned my B.Sc. in Engineering from the Free University of Brussels, in Belgium, and a Ph.D. in theoretical physics from Columbia University.

3. I have reviewed the instant application and the November 6, 2003 Office Action in this case.

4. Based on information and belief, it is my opinion that the claimed sequencing apparatus and substrate of the instant application are vastly superior to other sequencing systems, including the system reported by Chee et al. in Published Application No. U.S. 2003/0108867 ("Chee et al."), as cited by the Examiner.

The superior performance of the claimed invention can be attributed to the functional features of the apparatus and substrate, which include: 1) compact wafers; 2) attachable optical fibers; 3) flow chamber and fluid means; and 4) specific fiber and well sizes. As a result of these features (and others), the claimed invention provides the first massively parallel, solid-phase, whole-genome sequencing platform that can be scaled for viral, bacterial, and even human genomes.

Functional features of the claimed substrate and apparatus:
compact wafers can be placed in flow chambers for efficient fluid exchange

5. As recited in the claims, the compact wafers of the invention allow *placement into flow chambers*, which utilize an *efficient fluid exchange system* and thereby provide significantly faster sequence analysis. The advantages of the claimed apparatus and substrate are fully disclosed in the instant application, as filed.

6. The instant application teaches that the claimed apparatus and substrate include a compact wafer formed from a bundle of optical fibers, cut and polished to a thickness of 0.5 mm to 5 mm. This teaching is found, *inter alia*, in the originally filed application on p. 36, l. 12-15; and p. 36, l. 30 to p. 37, l. 3.

7. The instant application additionally teaches that the claimed substrate can be used with a flow chamber and fluid means for delivering sequencing reagents and washes to the wafer surface. This teaching is found, *inter alia*, in the originally filed application in Figs. 2 and 3; on p. 4, l. 14-18; p. 30, l. 15-19; p. 33, l. 20 to p. 35, l. 20; and in Exmp. 3 on p. 53, l. 28 to p. 54, l. 15.

8. The instant application further teaches that the underside of the compact wafer in the flow chamber can be optically linked or directly contacted with a fiber optic bundle to allow image capture, for example, through a CCD system. This teaching is found, *inter alia*, in the originally filed application on p. 34, l. 13-18; and Fig. 2.

9. The instant application teaches also that the underside of the compact wafer in the flow chamber can be placed in proximity to conventional optics mechanism, e.g., a high numerical aperture lens system to allow for image capture. This teaching is found, *inter alia*, in the originally filed application on p. 34, l. 19-23.

10. The claimed wafer for use in the flow chamber allows for much faster sequence analysis. The claimed apparatus and substrate thereby yield significantly improved results, which are not obtained with other sequencing systems such as that reported by Chee et al.

11. As cited by the Examiner, Chee et al. does not specify the length of the optic fibers for their sequencing system. Instead, Chee et al. relies on WO 98/50782 (see Chee et al., ¶ [0007]), which reports the use of optic fibers that are several meters long (Ex. 1). These long and bulky fibers cannot readily fit into a flow chamber. Instead, Chee et al. reports methods of inverting the long optic fiber and sequentially dipping the tip into individual cups filled with solutions of single nucleotides (Chee et al., ¶¶ [0192] – [0195], *inter alia*). This awkward dipping process is necessitated by the long, bulky fiber employed in Chee et al.

12. The dipping method of Chee et al. is predicted to be completely or partly inoperable. First, the continuous plunging of an optic fiber tip into nucleotide solutions would tend to dislodge any beads from the wells. Second, for DNA attached directly to wells, dipping would be ineffective in delivering the nucleotide solution to the wells due to the counteraction of air pressure. This phenomenon is generally observed for inverted cups or glasses placed into reservoirs of water, and provides the basis for the oceanographic apparatus known as the diving bell. An illustration provided as an aid in understanding is included as Ex. 2 (adapted from http://home.earthlink.net/~dmocarski/chapters/chapter7 /main.htm).

13. Even assuming, *arguendo*, that the dipping method of Chee et al. were marginally effective in delivering reagents and preserving samples for sequencing, the method would be extremely slow and inefficient compared to the claimed invention. The long fiber optic tip of Chee et al. would need to be inverted and dipped in and out of at least four cups (e.g., A, T, C, G), or perhaps more than eight cups (e.g., A, first wash, T, second wash, C, third wash, G, fourth wash) to determine only one nucleotide of sequence. In contrast, compact wafer of the invention can be placed into a flow chamber to allow for rapid and efficient delivery of sequencing reagents and washes to the compact wafer. An illustration provided as an aid in understanding is included as Ex. 3.

14. Because the long optic fibers of Chee et al. cannot readily fit into a flow chamber, the sequencing reactions are performed using an unwieldy dipping process. This results in significant delays and increased sample losses. By comparison, the claimed wafer is fitted into a flow chamber to allow streamlined processing and sequence analysis. This is a *significant functional advantage* over the system reported in Chee et al.

Functional features of the claimed substrate and apparatus:
compact wafers have well sizes to maximize signal capture and minimize sample loss

15. As recited in the claims, the compact wafers of the invention include *optimally sized fibers and wells* that allow *maximal signal capture and minimal sample loss* and thereby provide significantly improved sequence analysis. The advantages of the claimed apparatus and substrate are fully disclosed in the instant application, as filed.

16. The instant application teaches that the claimed apparatus and substrate include a compact wafer that includes optic fibers with a diameter of 3 μm to 100 μm. The application teaches that this diameter is important to ensure that each light signal can be captured as a single pixel. This teaching is found, *inter alia*, in the originally filed application on p. 36, l. 15 and 25-29.

17. The instant application teaches that the claimed apparatus and substrate include a compact wafer that includes wells with a depth of one-half to three times the diameter of the optic fibers. The application expressly recognizes the problem of bead/sample loss during the sequencing reaction. This teaching is found, *inter alia*, in the originally filed application on p. 37, l. 6-9; p. 39, l. 22-24; and Fig. 4.

18. The claimed fiber diameter and well depth of the compact wafer allow for much more effective sequence analysis. Accordingly, the claimed apparatus and substrate yield significantly improved results, which are not obtained with other sequencing systems, including the system reported by Chee et al.

19. As cited by the Examiner, Chee et al. ¶ [0105] apparently reports the use of long optic fibers with diameters ranging from approximately 0.17 μm to 0.03 μm. These diameters can be calculated from "high density" arrays indicated by Chee et al. ¶ [0105], i.e., arrays containing 40,000 fibers/mm^2 to 1,000,000 fibers/mm^2 (Office Action, quoting Chee et al. on pages 9-10). Chee et al. appears to be silent as to the specific well depths employed with the optic fibers.

20. With diameters of approximately 0.17 μm to 0.03 μm, many of the optic fibers employed by Chee et al. would produce sequencing systems that are completely or partly inoperable. Optic fibers having a such small diameters would require bead and well sizes less than 0.17 μm to 0.03 μm in diameter. Such systems would be predicted to have a myriad of problems, including difficulties in distinguishing light signals from each fiber and in depositing the beads in the wells. By comparison to Chee et al. ¶ [0105], the compact wafer of the invention employs optic fibers 3 μm to 100 μm in diameter to provide for maximal sample density while still allowing accurate signal detection and efficient bead delivery.

21. Chee et al. do not appear to specify well depths for use with the optic fibers, and evidently fail to recognize the importance of well depth in preventing sample loss.

In fact, Fig. 1 in Chee et al. shows beads and samples jutting out from their wells. This configuration would likely lead to significant sample loss during the "invert and dip" process reported by Chee et al. In contrast, the compact wafer of the invention employs well depths of one-half to three times the diameter of the fiber, which are important in minimizing sample loss during preparation and analysis. The optimally sized fibers and wells (¶¶ 16 and 17, above) therefore represent *significant functional advantages* over the system reported in Chee et al.

Superior function of the claimed substrate and apparatus:
massively parallel analysis of viral and human genomic sequences

22. As a result of these highly advantageous, functional features (¶¶ 14, 20, and 21, above), and other important aspects, the substrate and apparatus claimed in the instant application are the first to allow rapid massively parallel sequencing for whole genomes.

23. Traditional methods for genome sequencing have been slow, expensive, laborious, and industrial-scale, since they involve individually preparing and sequencing DNA fragments of the genome. The Human Genome Project, for example, required approximately 12 years, $2.7 billion dollars, and 60 million samples to complete.

24. In contrast, the substrate and apparatus claimed in the instant application provide a massively parallel, scalable platform that dramatically reduces the time, cost, sample preparation, and space required for genome sequencing. Instead of individually preparing and sequencing each sample, the claimed substrate and apparatus allow parallel sequencing of thousands (or hundreds of thousands) of samples.

25. Recently, the claimed substrate and apparatus were used to sequence the entire adenovirus genome (approximately 30,000 base pairs) contained on an expression vector in less than one day (see NY Times article, Ex. 4). The entire sequencing

process from sample preparation to data analysis was accomplished in less than one day, and provided over 99% genome coverage. The resulting adenovirus sequence was published in GenBank under Accession Nos. AY370909, AY370910, and AY370911 (Ex. 5).

26. In further experiments, the apparatus of the instant application was used to sequence a segment human chromosome 12 (approximately 170,000 base pairs) contained on an artificial chromosome vector (Ex. 6). With the apparatus, a one-day sequencing run produced sufficient shotgun sequence coverage of the chromosome 12 clone (Ex. 6, p. 6). A single sequencing run obtained 85% genome coverage and 98% consensus accuracy (Ex. 6, p. 3). These results were presented at the15th Annual Genome Sequencing and Analysis Conference, held on September 21-24, 2003 (Ex. 6, p. 1).

27. To generate this sequence information described in ¶¶ 25 and 26 (above), preferred commercial embodiments of the claimed substrate and apparatus were fabricated. In these preferred embodiments, the claimed substrates (termed "PicoTiter Plates") were formed from cavitated fiber optic wafers formed from a fused bundle of a plurality of individual optical fibers as taught and claimed by the instant application.

28. Specifically, PicoTiter Plates were made acid etching the top surface of fiber optic wafers to form wells with diameters between 39 and 44 μm, as currently claimed. The fiber optic wafer exhibited a thickness of about 2.0 mm, also as currently claimed. In addition, the wells on PicoTiter Plates were fabricated with depths ranging from 26 to 76 μm (i.e., from between one half the diameter of an individual optical fiber and three times the diameter of an individual optical fiber, as recited in the pending claims). Finally, the wells were loaded with nucleic acid template and beads with pyrophosphate sequencing reagents attached thereto, as recited in the pending claims. Sequencing by synthesis was then performed as

described in the specification, and using the claimed apparatus to flow sequencing reagents over the PicoTiter Plate.

29. The substrate and apparatus claimed in the instant application therefore fulfill a long-felt but unmet need for rapid, whole-genome analysis of viral and bacterial pathogens (e.g., ¶ 25, above). Such analysis is critical for biodefense, drug discovery, and the identification of emerging pathogens. More than this, the claimed apparatus solves the long-standing problems with analysis of large genomes, such as human genomes (e.g., ¶ 26, above). Solutions for large-genome sequencing are vital for drug development, early diagnosis, and faster clinical interventions.

30. For these reasons, in my opinion, the claimed substrate and apparatus represent a significant advancement in the field as the first massively parallel, solid-phase, whole-genome sequencing platform that can be scaled for the smallest to the largest genomes.

Conclusion

31. Therefore, based on information and belief, and all of the foregoing, it is my opinion the claimed sequencing apparatus and substrate substantially outperform the sequencing platforms used by Chee et al. and others. This is due to the functionally superior features of the claimed invention, which include compact fiber optic wafers, detachable fiber optic bundles, flow chambers and fluid means, and specifically sized fibers and wells. All of these features, and the other aspects of the invention, work together to achieve significantly faster results compared to other sequencing systems.

32. I declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18

U.S.C. § 1001 and that willful false statements may jeopardize the validity of this application and any patent issuing therefrom.

Dated: 4/20/04

Signed: _____
MARCEL MARGULIES

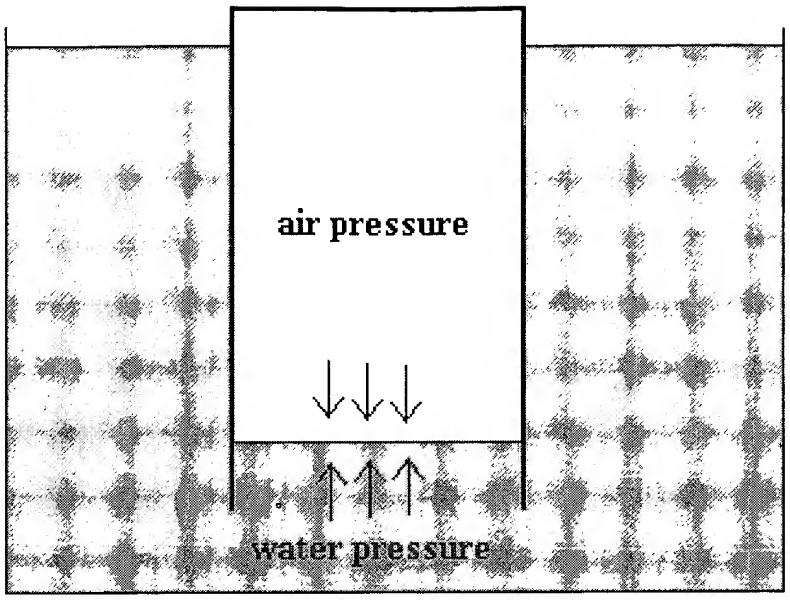
A. The Format Choices

The individually clad, optical fiber strand

A typical optical fiber strand is illustrated by Figs. 1 and 2A and 2B. As seen therein, an individual optical fiber strand 10 is comprised of a single optical fiber 12 having a rod-like shaft 14 and two fiber ends 16, 18, each of which provides a substantially planar end surface. The intended distal surface 20 at the fiber end 16 is illustrated by Fig. 2A, while the intended proximal surface 22 at the fiber end 18 is illustrated within Fig. 2B. It will be recognized and appreciated that the terms "proximal" and "distal" are relative and interchangeable until the strand is ultimately positioned in an apparatus. The optical fiber 12 is composed typically of glass or plastic; and is a flexible rod able to convey light energy introduced at either of its ends 16 and 18. Such optical fibers 12 are conventionally known and commercially available. Alternatively, the user may himself prepare individual optical fibers in accordance with the practices and techniques reported in the scientific and industrial literature. Accordingly, the optical fiber 12 is deemed to be conventionally known and available as such.

It will be appreciated that Figs. 1-2 are illustrations in which the features have been purposely magnified and exaggerated beyond their normal scale in order to provide both clarity and extreme detail. Typically, the conventional optical fiber has a cross section diameter of 5-500 micrometers; and is routinely employed in lengths ranging between meters (in the laboratory) to kilometers (in field telecommunications). Moreover, although the optical fiber 12 is illustrated via Figs. 1-2 as a cylindrical extended rod having substantially circular proximal and distal end surfaces, there is no requirement or demand that this specific configuration be maintained. To the contrary, the optical fiber may be polygonal or asymmetrically shaped along its length; provided with special patterns and shapes at the proximal and/or distal faces; and need not present an end surface which is substantially planar. Nevertheless, for best efforts, it is presently believed that the substantially cylindrical rod-like optical fiber having planar end surfaces is most desirable.

Each optical fiber 12 is desirably, but not necessarily, individually clad axially along its length by cladding 26. This cladding 26 is composed of any material with a lower refractive index than the fiber core and prevents the transmission of light energy photons from the optical fiber 12 to the external environment. The cladding material 26 may thus be composed of a variety of radically different chemical formulations including various glasses, silicones, plastics, platings, and shielding matter of diverse chemical composition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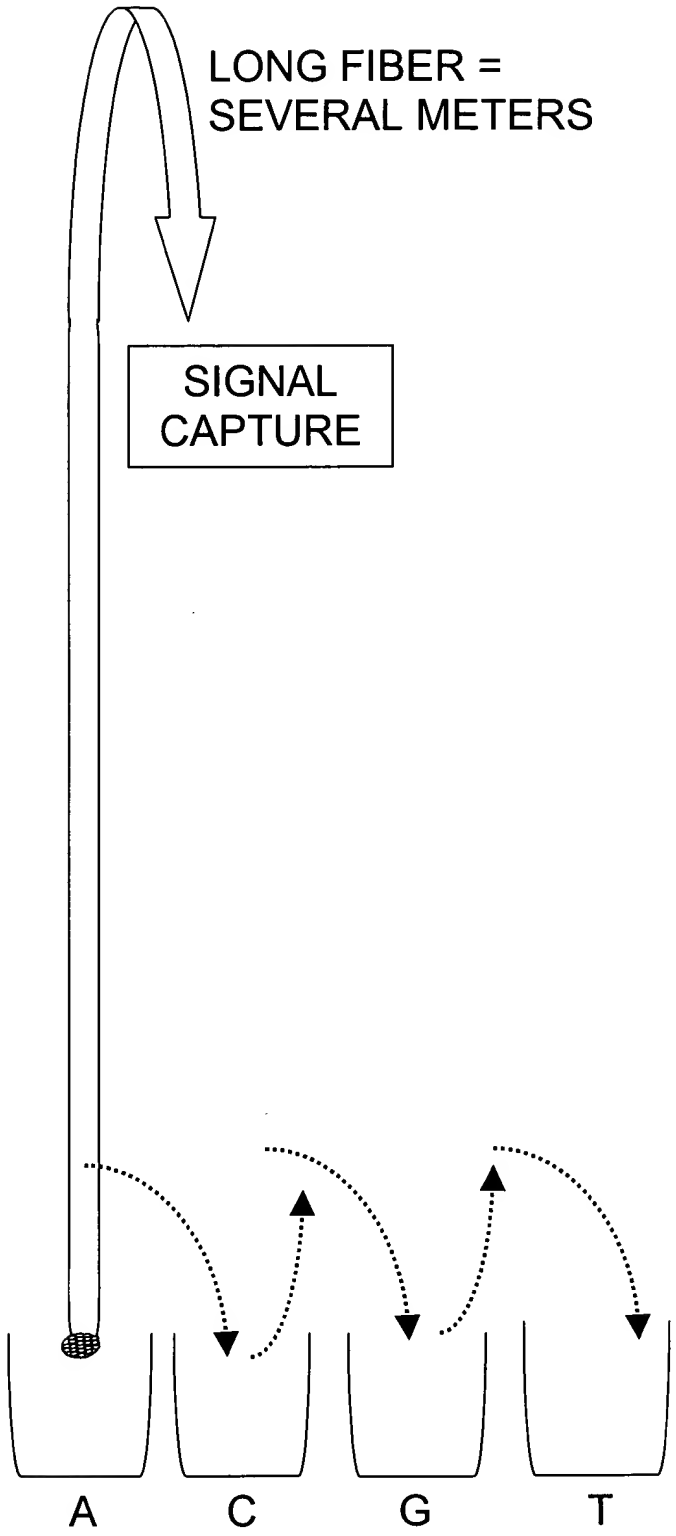
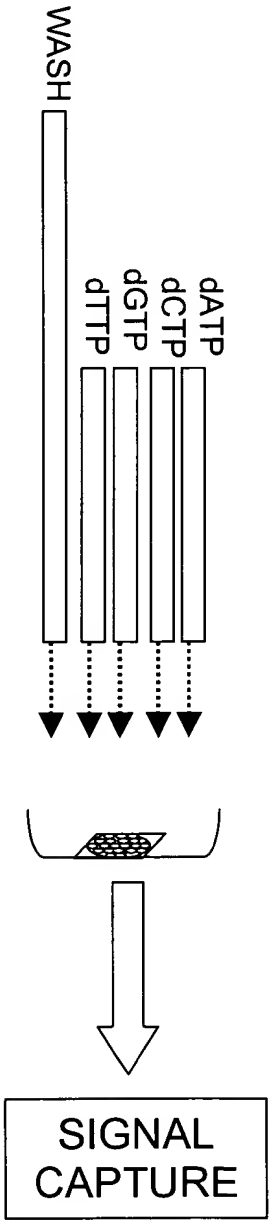
CHEE et al.

CLAIMED SUBSTRATE





CANNOT USE FLOW CHAMBER – REQUIRES SLOW, WASTEFUL DIPPING PROCESS

CAN USE FLOW CHAMBER – ALLOWS FAST & EFFICIENT PROCESSING

Company Says It Mapped Genes of Virus in One Day

By ANDREW POLLACK

A small company developing a novel method of sequencing genes said yesterday that it had determined the entire genetic code of a virus in a single day.

The company, 454 Life Sciences, said it was the first time that the entire genome of an organism had been sequenced using an unconventional technique and demonstrated the feasibility of its technology, which it said might eventually allow gene sequencing to be done faster and less expensively.

"It's a real threshold moment," said Richard Gibbs, director of the genome-sequencing center at Baylor College of Medicine in Houston and an adviser to the company. "This is going to be big."

Other scientists were more skeptical, saying that sequencing a virus, which has a tiny genome, is trivial and that there are aspects of the

technology that might make it difficult to do more complex organisms.

"I think doing a whole bacterium will be a challenge," said Edward M. Rubin, director of the Joint Genome Institute, a Department of Energy sequencing center in Walnut Creek, Calif. Bacteria are the next level up in complexity after viruses.

454, based in Branford, Conn., is one of several companies racing to improve gene sequencing. Some scientists say it might one day be possible to sequence an entire human genome in a few days for as little as $1,000 so that each person could have his or her genome for use in predicting susceptibility to disease and choosing the most appropriate medicines. The Human Genome Project, which first sequenced a human genome, took years and cost tens of millions of dollars.

Still, the thousand-dollar genome is years away. The human genome, which consists of more than three billion letters of the genetic code, is about 100,000 times the size of the adenovirus that 454 sequenced, which has a genome of about 33,000 letters.

454 is majority owned by CuraGen, a genomics company. The name 454 was the code name by which the project was referred to at CuraGen and the numbers have no special meaning, Richard F. Begley, chief executive of 454, said. Some of the company's technology is licensed from Pyrosequencing A.B., a Swedish company. Mr. Begley said the company hoped to start offering a sequencing service by the end of the year and to begin selling machines about six months after that.

Sequencing is usually done in big sequencing centers with dozens of machines and dozens of people. Dr. Begley said 454's technology would pave the way for the "personalized genome center" in which a single scientist could do sequencing on one

machine in a small room.

The company's technique does more sequencing in parallel than the conventional technique, he said. Another significant time-saving development, he said, comes in preparing the samples for sequencing. The 454 technique, he said, requires one sample preparation for each organism compared with dozens and even thousands for the conventional technique.

Scientists differed on whether sequencing a virus in a day was truly fast. The Department of Energy once did 30 bacteria in 30 days. The virus that causes SARS was sequenced in six days, but it might be misleading to compare that effort with 454's.

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  AUTHORS   Lohman,K.
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            Norton,W., Reifler,M., Rodgers,G., Ronan,M., Simpson,J.,
            Tartaro,K., Verma,S., Zimmerman,Z., Dacey,P., Begley,R. and
            Lohman,K.
  TITLE     Sequence Analysis of the pAdEasy-1 Recombinant Adenoviral Construct
            Using the 454 Life Sciences Sequencing-by-Synthesis Method
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1062)
  AUTHORS   Lohman,K.
  TITLE     Direct Submission
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            Lohman,K.
  TITLE     Sequence Analysis of the pAdEasy-1 Recombinant Adenoviral Construct
            Using the 454 Life Sciences Sequencing-by-Synthesis Method
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 30091)
  AUTHORS   Lohman,K.
  TITLE     Direct Submission
  JOURNAL   Submitted (18-AUG-2003) 454 Life Sciences, 20 Commercial Street,
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Human BAC Sequence and Assembly by 454 Life Sciences' Sequencing by Synthesis Method

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[1] 454 Life Sciences, Branford, CT

[2] Human Genome Sequencing Center, Baylor College of Medicine, Houston, TX

454 LIFE SCIENCES

Abstract

454 Life Sciences has developed proprietary methods for massively parallel DNA sequencing. We have applied this technology to re-sequencing and mapping human BAC clones to their precise chromosomal locations. This preliminary data shows the efficacy of the technology to rapidly sample and characterize subsets of sequence spanning an entire genome or a specific chromosomal location. The novel DNA sequencing method consists of three steps: template preparation, solid phase amplification and solid phase DNA sequencing. Several thousand to several hundreds of thousands of DNA sequencing reactions are performed simultaneously on glass plates containing 300 thousand to 1 million, 75 picoliter volume wells. Average read length of each fragment is consistently greater than 50 bases. The starting point for genome sequencing involves a single-tube template preparation and an absence of a bacterial plasmid cloning step, thus greatly reducing costs and increasing the throughput of our system. In addition, we are completing development of a new software algorithm for de novo whole genome assembly. Sequencing results from human BAC clones will be presented and discussed.

Experimental Procedures

Our novel methodology requires only a single sample preparation per genome, utilizes simultaneous clonal amplification of shotgun fragments in sub-nanoliter microreactors, without the use of time-consuming cloning steps. The product of each microreactor is driven to and captured by a concomitant solid support. The captured DNAs are delivered to wells on the PicoTiterPlate™ and sequenced on 454 Life Sciences sequencing platform. The details of these steps are illustrated in Figure 1.

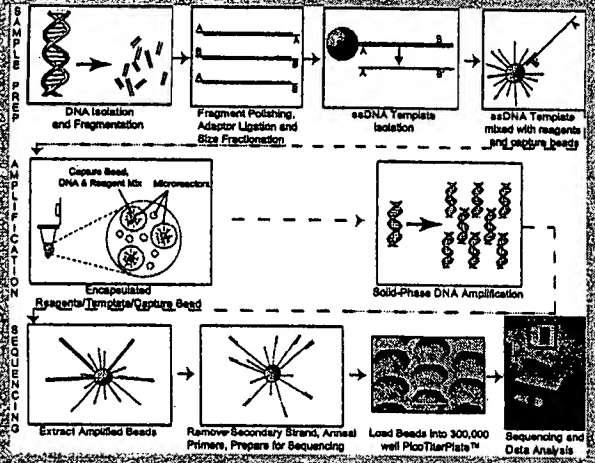

Figure 1: Streamlined template preparation and amplification process.

1) BAC DNA from clone RP11-418C2 was fragmented to sub-kilobase lengths.
2) The fragment ends were polished, 5' and 3' adaptors ligated onto each fragment, and the sample was size fractionated, resulting in products under 500 bases in length.
3) One strand of these double-stranded products was bound to microparticles, and the free strand was eluted as template for the subsequent amplification reaction.
4) Amplification was conducted in a single-reaction preparation, encapsulating the reaction reagent mix, a single DNA capture bead, and template in a 40 to 100 picoliter microreactor.
5) The particular template molecule contained in each individual microreactor was amplified and immobilized on the respective DNA capture bead.
6) The DNA capture beads were extracted and the template DNA was prepared for use on the 454 sequencer.

Data Analysis

The 454 sequencer generates raw traces for each microreactor and produces sequence reads in FASTA format using a proprietary basecaller program. Adaptors and low quality reads are removed and repeats masked before mapping and assembly.

Human Genome Mapping:
Each masked read was mapped against the human genome (NCBI build 33) using BLAT and the mapped reads (>95% identity) are recorded for each chromosome.

BAC Assembly:
Each sequence was mapped against the reference BAC sequence (RP11-418C2) using a proprietary alignment algorithm and the resulting alignment was recorded. For sequences that map to the genome with >90% accuracy, the software generates a list of individual bases found at a given position in the reference genome. The consensus base for each location was computed by averaging all mapped bases. This consensus sequence was then compared with the reference sequence to calculate total accuracy and coverage.

We also mixed 3x oversample of reads (950 sequences) generated from conventional Sanger method with reads generated from the 454 sequencer and assembled with Phrap using default parameters.

Results and Discussion

Human Genome Mapping:
Out of 8561 mapped reads, 7153 are mapping to human chromosome 12 (Fig. 2a). Of these, 7058 reads map to the expected location within chromosome 12 (Fig. 2b). The coordinate boundaries for clone RP11-418C2 in NCBI build 33 are 11,818,492 to 11,986,440, whereas boundaries on the 7058 read stack are 11,816,616-11,986,514. We also mapped these reads to the mouse genome, and located the BAC to the syntenic region on mouse chromosome 6 (data not shown).

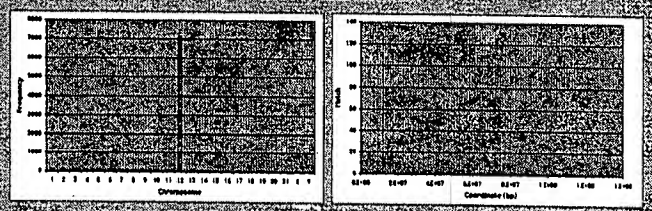

Figure 2: Sequence mapping against human genome and within chromosome 12.

BAC Assembly:
In a separate sequencing run, we generated 57,193 raw reads from this BAC clone. After adaptor removal, repeat masking, and quality trimming, 39900 reads were assembled against the reference sequence (Fig. 3). Genome coverage is 85% and consensus accuracy is 98%. Average read length is 84 bases.

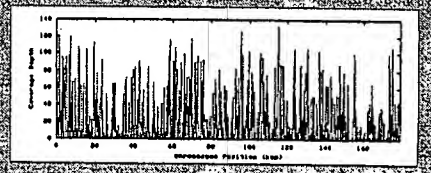

Figure 3: Frequency of assembled reads across BAC sequence length.

Phrap Assembly:
Sanger reads alone generated 25 major contigs (>2 kb) with a 76% mapping efficiency, whereas Sanger and 454 reads combined produced 18 major contigs with a 83% mapping efficiency. 454 reads were able to join and extend Sanger contigs into much larger stretches (Fig. 4).

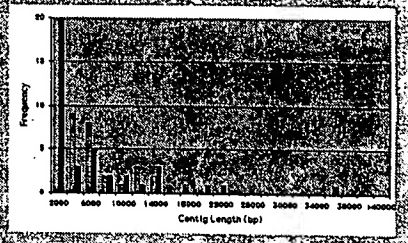

Figure 4: Distribution of Phrap contigs from Sanger only and Sanger+454 reads.

Conclusion

We have demonstrated in this study that 454's Life Sciences' novel sequencing methodology is capable of producing sufficient shotgun sequence coverage of a BAC clone in a single run (done within 1 day). The reads can be used to map its precise location in the genome, as well as assembling into contigs based on a reference sequence. This is a useful tool for whole genome mapping and sequencing.

We also showed that by combining conventional Sanger method with 454 technology, we achieve a better *de novo* assembly outcome for whole genome shotgun sequencing.

We are continuing to develop our quality scoring and trimming algorithm. We have completed phase one of our proprietary fragment assembler, designed to take advantage of the raw trace signals produced by our sequencing-by-synthesis method. This assembler will be available as part of 454's commercial sequencing instrument.

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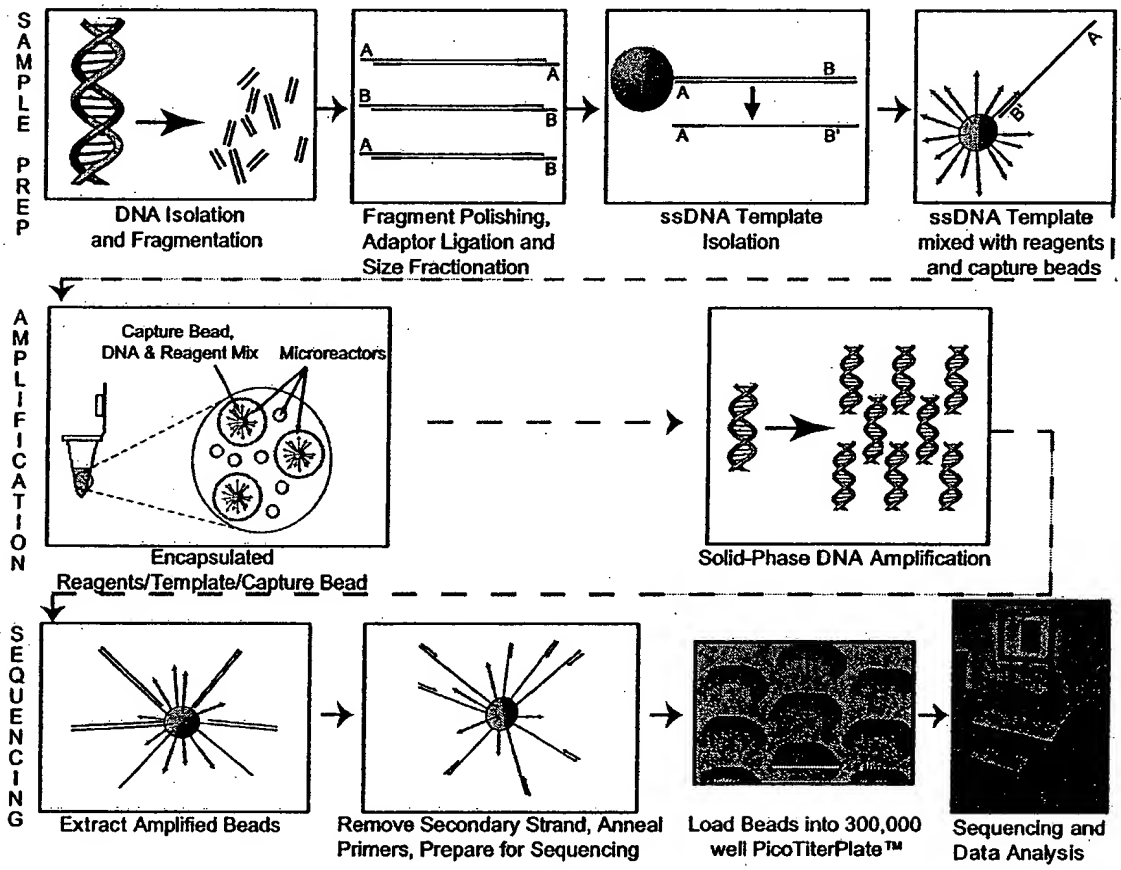

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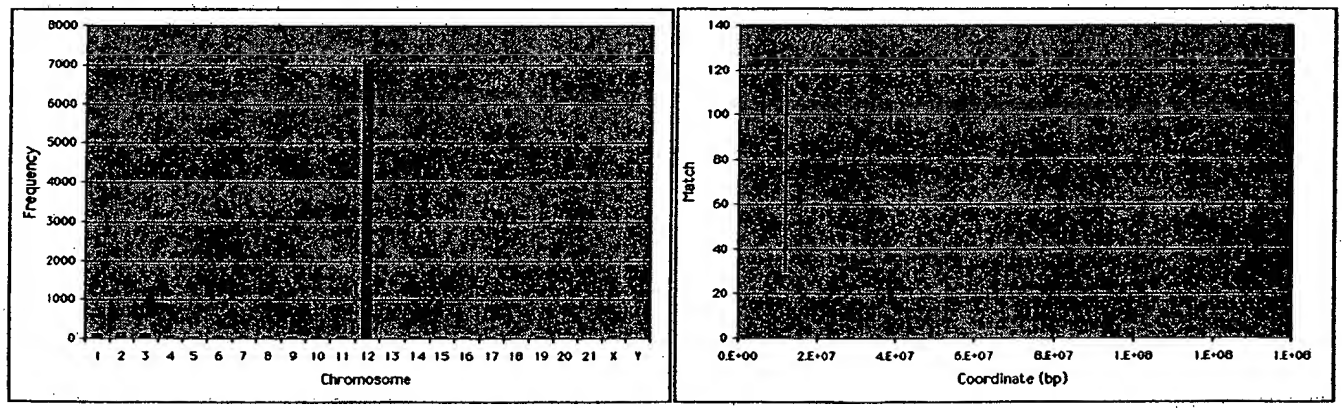

(a) Mapping against Human Genome (b) Mapping to Human Chr12

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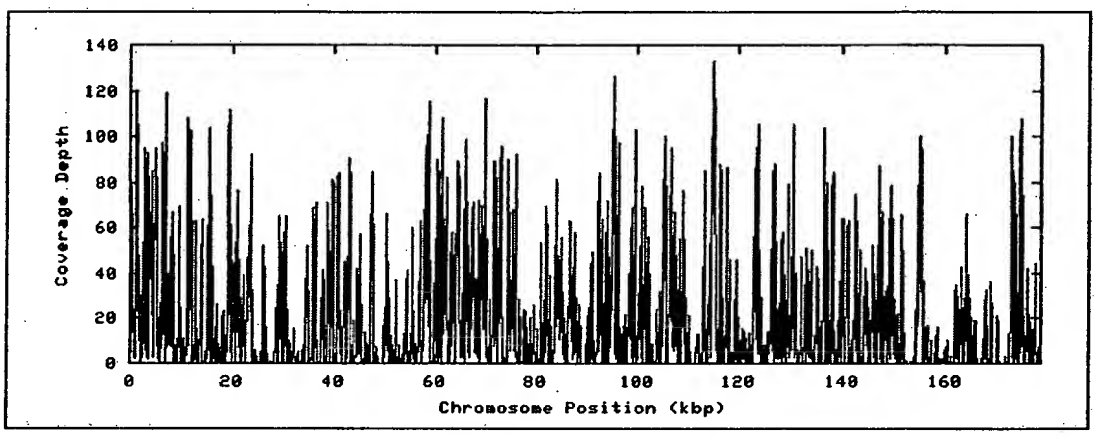

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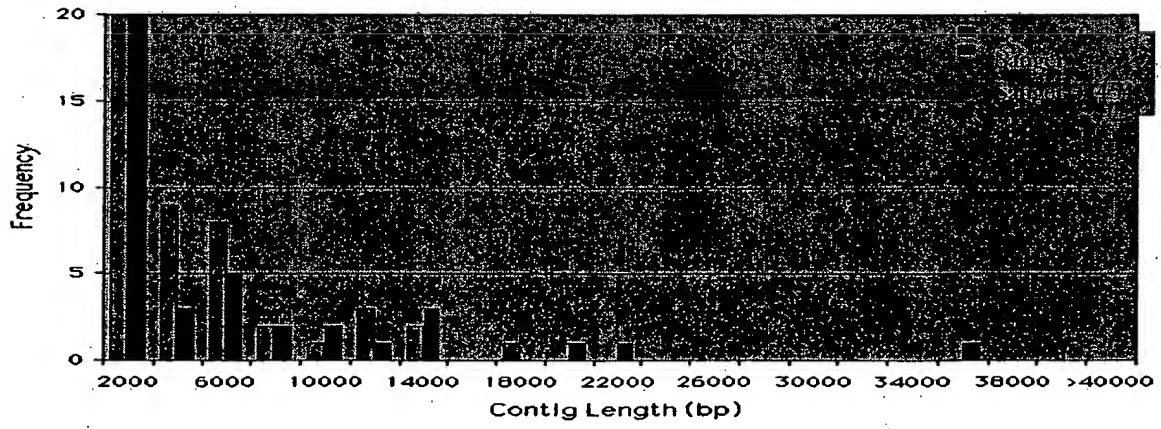

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